



Photodynamic Inactivation of Viruses: The Factors that Determine Rate and Extent of Inactivation

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ABSTRACT

Photodynamic inactivation (PDI) of viruses in solution has been reported and that singlet oxygen mediated oxidation is responsible for the inactivation. The capsids of non-enveloped viruses are possible targets for viral inactivation by singlet oxygen oxidation. In most of the previous PDI studies, much of the work has been focus on the quantitative aspects of the inactivation with little emphasis on the factors and targets of the PDI in viruses. In this work, we carried out the PDI of our model virus-bacteriophage MS2 using different concentrations of photosensitizer-TMPyP in solution, different light intensities and at different times of light exposure (illumination). The results obtained in this study suggest that the concentration of the photosensitizer- TMPyP, time of illumination and light intensity all affect the rate and extent of PDI of MS2. Also, we observed that 0.1% (w/v) of humic acid gave 100 % protection to MS2 under the PDI conditions used. A plausible explanation for this include the humic acid quenched the singlet oxygen generated or shielded the virus from direct effect of singlet oxygen mediated damage. This work can lead way in providing needed information on all factors that should be considered when developing/establishing an optimal PDI conditions for the control of viruses.

Keywords: MS2, singlet oxygen, TMPyP, photodynamic inactivation, humic acid

INTRODUCTION

Photodynamic inactivation (PDI) of viruses has been shown to be a promising alternative antiviral agent for controlling viruses (Casteel *et al.*, 2004, Silverman *et al.*, 2013, Wainwright, 2004, Costa *et al.*, 2009, Costa *et al.*, 2008, Costa *et al.*, 2010). PDI is a process in which the resultant singlet oxygen and other reactive oxygen species (ROS) from photodynamic effect oxidise and cause irreversible damage to proteins, lipids, nucleic acid and other cellular components of microorganisms and ultimately inactivates them (Costa *et al.*, 2013). The photodynamic effect resulted from the interaction of three factors comprising of photosensitizer, visible light and oxygen (Costa *et al.*, 2013). PDI is an indirect photochemical reactions-also called photosensitized reaction which arose from the possibility that the energy of the excited photosensitizer molecule at triplet state can be transferred to another molecule such as oxygen by a non-radiative mechanism (DeRosa and Crutchley, 2002, Costa *et al.*, 2013). The transfer can occur either by a charge transfer

mechanism (type 1 photoreaction) leading to production of hydroxyl radical, superoxide anion, peroxide and hydroxyl ion or by electronic energy transfer mechanism (type 2 photoreaction) leading to production of singlet oxygen (Costa *et al.*, 2013, DeRosa and Crutchley, 2002). These transfers inhibit emission of radiation by the excited photosensitizer molecule and this is called quenching (DeRosa and Crutchley, 2002). It is believed that singlet oxygen is responsible for the photodynamic inactivation of viruses (Costa *et al.*, 2013). Among biomolecules, protein has the highest bimolecular rate constant of singlet oxygen mediated oxidation (10^5 to 10^9 $M^{-1} S^{-1}$) followed by RNA (10^4 to 10^6 $M^{-1} S^{-1}$) (Davies, 2003, Cho *et al.*, 2010). Singlet oxygen and other reactive oxygen species can only cause damage to nearer surrounding molecules because of their high reactivity and short half-life (Costa *et al.*,

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2013).

Several studies have been reported about PDI of viruses in solution. Many of the PDI studies have revealed that the rate and extent of PDI of viruses is proportionally dependent on the concentration/dose of photosensitiser and time/duration of PDI. However, there are only few investigations of how other factors such as source of light, light intensity, co-pollutant and virus itself could affect the PDI. Costa *et al.*, (2008 and 2010) have shown how net charge of a photosensitiser and light parameters affect rate and extent of PDI of phage T4-like. It was reported that tetra- and tri- cationic porphyrins are more efficient than di-cationic porphyrins in PDI of the phage T4-like. Also, when same light source with a fixed dose was applied at different fluence rates, it was observed that the inactivation of phage T4-like with tetra- and tri- cationic porphyrins in solution was significantly higher when low fluence rates were used (Costa *et al.*, 2010). We believed that for PDI to be successfully used as an efficient alternative antiviral agent, there is a need for systematic investigations of all factors of PDI in order to have complete knowledge of what to consider when establishing an optimal PDI protocol/system for the control viruses.

Bacteriophage MS2 was used as a viral model in this study. MS2 has been used as a viral model organism in several studies aimed at photoinactivation and chemical disinfection of human viruses because of its similarity in size and morphology to some human viruses such as noroviruses and picornaviruses (Kohn and Nelson, 2007, Zhong *et al.*, 2016). Also, because it is non-pathogenic to human, so poses no health risk and easy to propagate. In this work, PDI of the model virus was investigated with different concentrations of TMPyP in solution, different light intensities and at different times of light exposure (illumination).

METHODOLOGY

Light source, photosensitiser-TMPyP, virus strain and host bacteria cells

A Schott KL 2500 LCD (Schott Ltd., Stafford, UK), which provides a cool white light in the visible region of the spectrum was applied as the light source for the PDI experiments. Fluence rates of illumination during photoinactivation experiments were measured using a light meter (Clas Ohlson, UK).

Photosensitiser-TMPyP was purchased without further purification from Sigma Aldrich. Absorption spectra of TMPyP (Figure 1) in phosphate buffered saline (PBS) (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl) was determined using a NanoDrop 2000_C micro-spectrometer (Thermo Scientific) at 20-22 °C under aerobic conditions. Stock of bacteriophage MS2 and the *E. coli* host cells were a gift from Prof. P. Stockley University of Leeds, UK.

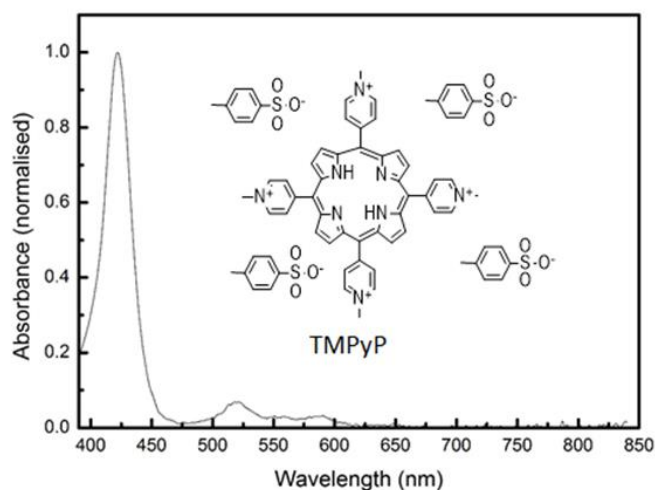


Figure 1: Absorption spectra of the photosensitiser-TMPyP used for PDI of MS2. The absorption peak of TMPyP is 422 nm. The TMPyP chemical structure was from ChemACX.com, ChemDraw Pro 13.0.

Propagation and purification of bacteriophage MS2

The bacteriophage MS2 was propagated in *E. coli* and purified as previously described (Majiya *et al.*, 2018).

Photodynamic inactivation of MS2

PDI of the model virus was investigated with different concentrations of TMPyP in solution, different light intensities and at different times of light exposure (illumination). The different concentrations of TMPyP used were 0.1 μM to 50 μM, different light intensities used were 5 mW cm⁻² to 466 mW cm⁻² and different time of illuminations were 10 sec to 120 min. As controls, dark experiments (D) were carried out in the presence of photosensitizer but without illumination, while “no sensitiser” (NS) experiments were exposed to light but in the absence of photosensitiser. All experiments were repeated in triplicate.

Infectivity and titre determination of model viruses

The double layer agar plaque assay (Kropinski *et al.*, 2009) was used to determine the infectivity and titre of bacteriophages MS2 after purification and before and after PDI to know the rate and extent of PDI.

Statistical and graphical software

Data from different experiments were imported to Microsoft Excel. All graphical outputs were plotted in Origin Pro 8. The chemical structure was drawn using ChemDraw Pro v13.0.

RESULTS AND DISCUSSION

Effects of concentration of TMPyP and illumination time on PDI of MS2

The dependency of the extent of PDI on TMPyP concentration/dose and illumination time were investigated in solution. PDI of MS2 was investigated at constant visible light intensity of 32 W.cm^{-2} with different concentrations of TMPyP in solution and at different times of illumination. This light intensity (32 W.cm^{-2}) is low and is about 10% mid-day but allows a graded response to be shown. Double layer agar plaque assay was used to carry out viability test and to determine the extent of PDI in MS2.

Our data showed that TMPyP with concentration of at least $0.2 \mu\text{M}$ in solution caused complete inactivation of MS2 within 60 sec when illuminated at 32 W.cm^{-2} (Figure 2 A). At 10 seconds of illumination there were

1.5 log reductions in PFU/ml (Figure 2 B) and at 30 seconds of illumination there were 4 log reductions in PFU/ml of MS2 (Figure 2 B). There was no significant difference between the rate and extent of PDI in MS2 caused by $0.2 \mu\text{M}$ TMPyP (Figure 2 A) as compared to that caused by higher concentrations such as $50 \mu\text{M}$ TMPyP (Figure 2 C). In fact, complete MS2 inactivation was observed at 50 sec of PDI when $0.5 \mu\text{M}$ TMPyP was used (Figure 2 B) while it was 60 sec when $50 \mu\text{M}$ TMPyP was used (Figure 2 C). However, only the reductions of 4 log PFU/ml of MS2 was observed when $0.1 \mu\text{M}$ TMPyP was used for 60 sec PDI (Figure 2 A). TMPyP alone in the dark or light alone without sensitiser do not cause any detectable reduction in log PFU/ml of MS2 (Figure 2). Previous work has shown that tetra-porphyrins like TMPyP can efficiently inactivate bacteriophages such as phage T4-like in solution (Costa *et al.*, 2008, Costa *et al.*, 2010). It was reported that complete inactivation phage T4-like from sewage was achieved only at the highest TMPyP concentration used, $5 \mu\text{M}$, and illuminated at 40 W.m^{-2} for 270 minutes. Complete inactivation within 1 minute was also reported but at higher concentrations of 1 mM and $10 \mu\text{M}$ of TMPyP illuminated at 2.2 mW.cm^{-2} with a UV lamp (Casteel *et al.*, 2004). In this work, we avoided using a UV lamp as source of light and used cold visible light source instead, as UVC can inactivate microorganisms directly.

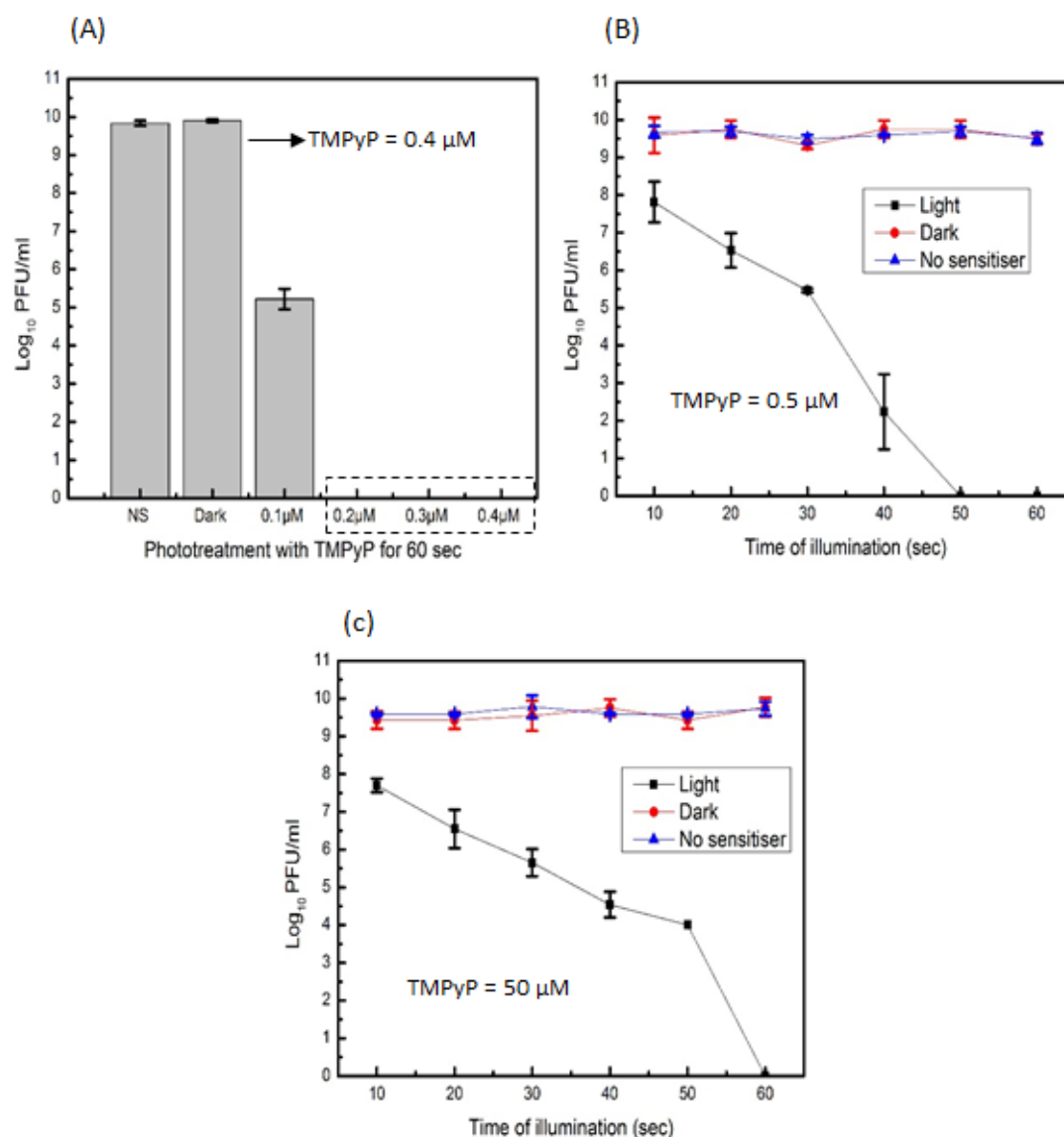


Figure 2: MS2 PDI in solution. Phage were illuminated at 32 mW.cm^{-2} .

(A), PDI using different concentrations ($0.1 \mu\text{M}$ to $0.4 \mu\text{M}$) of TMPyP; (B), PDI using $0.5 \mu\text{M}$ TMPyP in solution from 10 to 60 sec; (C), PDI using $50 \mu\text{M}$ TMPyP in solution from 10 to 60 sec. The dark controls were treated with the concentration of photosensitiser shown but not illuminated whilst no photosensitiser controls (NS) were illuminated without photosensitiser present. Data are mean \pm standard deviation (n = 3).

Effects of concentration of TMPyP and illumination time on PDI of MS2

Even though our data has shown that the illumination time has a proportional relationship with the rate and extent of PDI of MS2 (Figure 2), the effect of light intensity on PDI of MS2 was also investigated. PDI of MS2 was investigated at a constant concentration of $0.5 \mu\text{M}$ of TMPyP in solution and 60 sec illumination but at different light fluences. The different light intensities used were from 5 mW.cm^{-2} to 40 mW.cm^{-2} .

Also, we carried out experiments to determine the effect of co-pollutants on the rate and extent of PDI in MS2. We used PDI conditions ($0.5 \mu\text{M}$ photosensitiser, 32 mW.cm^{-2} and 30 sec and 60 sec of illumination) which caused reductions of $4 \log_{10}$ PFU/ml and complete inactivation of MS2 for 30 sec and 60 sec of illumination respectively (Figure 2). However, to test the effect of organic pollutants on PDI of MS2, in addition to these conditions the PDI was carried out in PBS that contained 0.1% (w/v) of humic acid.

In each PDI light experiment i.e. with photosensitiser present was carried out with two controls. These were a dark control, with photosensitiser but not illuminated and no sensitiser but still illuminated. Double layer agar plaque assay was used to carry out viability test and to determine the extent of PDI in MS2.

Our data showed that 20 mW.cm^{-2} , about 1.8 % of mid-day sunlight in sub-Saharan Africa, could achieve complete inactivation i.e. reductions of 9.5 log PFU/ml of MS2 PDI for 1 min using $0.5 \mu\text{M}$ TMPyP (Figure 3). We observed 3, 4 and 6 log reductions of PFU/ml of MS2 for the light intensities of 5, 10 and 15 mW.cm^{-2} respectively for 1 min MS2 PDI, also using $0.5 \mu\text{M}$ TMPyP (Figure 3). This indicates that there is proportional relationship between the light intensity and extent of PDI of MS2. It has been reported that using tetra and tri cationic porphyrins as photosensitisers, the extent of PDI of phage T4-like depends on the type of sensitiser and its concentration, light source, energy dose and fluence rate (Costa *et al.*, 2010). They observed that when the same light source and a fixed light dose was applied at different fluence rates, phage inactivation was significantly higher when low fluence rates were used (Costa *et al.*, 2010). This contradicts our findings at least for the light intensities from 5 mW.cm^{-2} to 20 mW.cm^{-2} where we observed proportional inactivation of MS2 (Figure 3).

Our data also showed that 0.1% (w/v) of humic acid in solution gave 100% protection to MS2 during PDI (Figure 4). This was at least true for the PDI conditions used ($0.5 \mu\text{M}$ photosensitiser, 32 mW.cm^{-2} and 30 sec and 60 sec of illumination). No MS2 inactivation was observed. (Figure 4). This concentration [0.1% (w/v) equivalent to 1 mg/ml] of humic acid gave dark chocolate colour to PDI solution leading to reduction of light transmittance and therefore molecules of photosensitiser-TMPyP were not excited or few were excited and ultimately no or very few singlet oxygen and other ROS were generated. It is also possible that the humic acid quenched the singlet oxygen and other ROS generated or shield the virus from direct effect of singlet oxygen mediated oxidation. However, some studies have shown that constituents (dissolved and particulate) from stabilisation ponds as well as synthetic humic acid and

natural humic acid extracted from a river can act as photosensitisers when exposed to full spectrum sunlight thereby inactivating MS2, adenovirus type 2 and bacteriophage PRD1 (Kohn and Nelson, 2007, Silverman *et al.*, 2013). It was reported that the efficiency of these photosensitisers at inactivating MS2 was in order; synthetic humic acid > natural humic acid extracted from a river > constituents of a stabilisation pond (Kohn and Nelson, 2007).

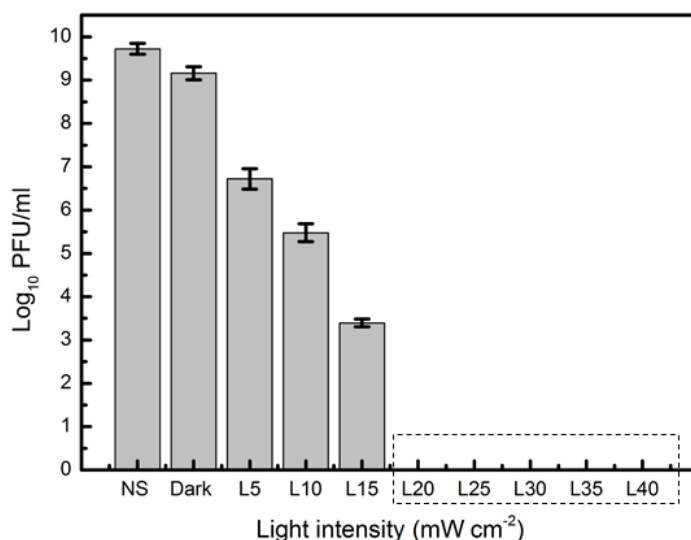


Figure 3: PDI of MS2 using $0.5 \mu\text{M}$ TMPyP in solution. Phage were illuminated for 60 sec at different light intensities. L5 to L40 indicates fluence rates of 5 mW.cm^{-2} to 40 mW.cm^{-2} . The dark controls were treated with the concentration of photosensitiser shown but not illuminated whilst no photosensitiser controls (NS) were illuminated without photosensitiser present. Data are mean \pm standard deviation ($n = 3$).

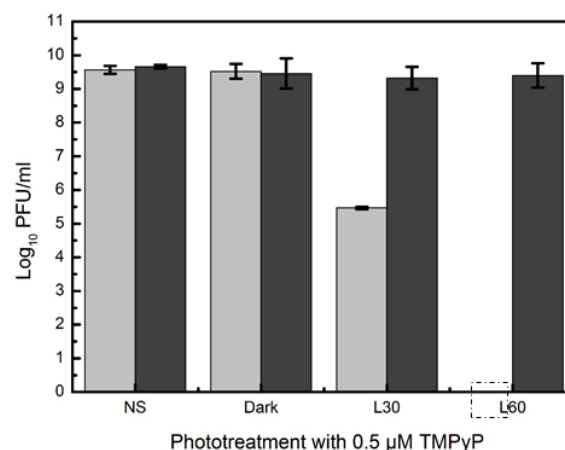


Figure 4: PDI of MS2 in the presence and absence of humic acid. Phage were

illuminated at 32 Mw.cm⁻². L30 and L60 indicates 30 sec and 60 sec illumination times respectively. The dark controls were treated with the concentration of photosensitiser shown but not illuminated whilst no photosensitiser controls (NS) were illuminated without photosensitiser present. Data are mean \pm standard deviation (n = 3).

(■), No humic acid; (■), 0.1% (w/v) humic acid

(□), Complete inactivation

CONCLUSION

The data obtained in this study showed that the concentration of the photosensitiser- TMPyP, time of illumination and light intensity all affect the rate and extent of PDI of MS2 in a dose dependent manner. Also, we observed that 0.1% (w/v) of humic acid gave 100 % protection to MS2 under the PDI conditions we used, although higher light intensity may reduce this. A plausible explanation for this include the humic acid quenching the ROS generated or shielding the virus from direct effect of singlet oxygen mediated damage. It is very possible also that because at this concentration of humic acid gave dark brown colour to the PDI solution and light transmittance through the solution was drastically reduced, in turn reducing ROS production. At higher light intensity and longer duration of illumination, inactivation may be observed.

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